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Tian, Hailin

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Address: DTU Environment
Department of Environmental Engineering
Technical University of Denmark
Miljøevej, building 113
2800 Kgs. Lyngby
Denmark

Phone reception: +45 4525 1600
Fax: +45 4593 2850

Homepage: http://www.env.dtu.dk
E-mail: reception@env.dtu.dk

Cover: GraphicCo

Preface

This Ph.D. thesis, entitled “Innovative bioaugmentation strategies to alleviate ammonia inhibition in anaerobic digestion process” comprises the research carried out at the Department of Environmental Engineering, Technical University of Denmark from October 1st, 2015 to September 30th, 2018. Professor Irini Angelidaki and Associate Professor Ioannis Fotidis were the main supervisor and co-supervisor, respectively.

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-VII**.

I **Tian, H.,** Treu, L., Konstantopoulos, K., Angelidaki, I., Fotidis, I.A. 16s rRNA gene sequencing and radioisotopic analysis reveal the composition of ammonia acclimatized methanogenic consortia. *Submitted to Bioresource Technology*, 02 Aug 2018.

II **Tian, H.,** Fotidis, I.A., Mancini, E., Angelidaki, I., 2017. Different cultivation methods to acclimatise ammonia-tolerant methanogenic consortia. *Bioresource Technology* 232, 1-9.

III **Tian, H.,** Fotidis, I.A., Mancini, E., Treu, L., Mahdy, A., Ballesteros, M., González-Fernández, C., Angelidaki, I., 2018. Acclimation to extremely high ammonia levels in continuous biomethanation process and the associated microbial community dynamics. *Bioresource Technology* 247, 616-623.

IV **Tian, H.,** Mancini, E., Treu, L., Angelidaki, I., Fotidis, I.A. Bioaugmentation strategy for overcoming ammonia inhibition during biomethanation of microalgae. *Submitted to Renewable Energy*, 22 Aug 2018.

V **Tian, H.,** Yan, M., Treu, L., Fotidis, I.A., Angelidaki, I. Bioaugmentation as a trigger for the establishment of an efficient microbiota: focus on ammonia inhibition in thermophilic anaerobic digestion process. (*Manuscript under preparation for submission*)

VI **Tian, H.,** Karachalios, P., Angelidaki, I., Fotidis, I.A., 2018. A proposed mechanism for the ammonia-LCFA synergetic co-inhibition effect on anaerobic digestion process. *Chemical Engineering Journal* 349, 574-580.

VII **Tian, H.,** Fotidis, I.A., Kissas, K., Angelidaki, I., 2018. Effect of different ammonia sources on aceticlastic and hydrogenotrophic methanogens. *Bioresource Technology* 250, 390-397.

In this online version of the thesis, paper **I-VII** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøevej, Building 113, 2800 Kgs. Lyngby, Denmark, info@env.dtu.dk.

In addition, the following publications, not included in this thesis, were also concluded during this PhD study:

Mancini, E., **Tian, H.,** Treu, L., Angelidaki, I., Fotidis, I.A. Energy-rich industrial wastewaters as potential biomethanation feedstocks. (*Manuscript under preparation for submission*)

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This PhD thesis is dedicated to everyone who has been by my side in the last three years.

Summary

Anaerobic digestion (AD) is a widely used biotechnology to recover energy (in the form of biomethane, CH_4) from various biowastes and biomasses. In recent years, nitrogen-rich substrates, such as chicken manure, slaughterhouse waste, microalgae, etc. are becoming attractive AD substrates due to their high methane potential. However, high ammonia levels caused by the degradation of these nitrogen-rich substrates usually inhibit the AD process, resulting in methane production loss and volatile fatty acids (VFAs) accumulation. In this Ph.D. project, an efficient bioremediation method, i.e. bioaugmentation, was developed to overcome the ammonia inhibition in the AD process and thereby improving the methane production.

First of all, the prerequisite of a successful bioaugmentation is to acquire the ammonia-tolerant methanogenic consortia and use them as bioaugmentation inocula. Thus the possibility of acclimatizing microbial community to high ammonia levels was assessed in this study. The results showed that microbial inocula taken from two mesophilic and three thermophilic full-scale biogas plants were successfully acclimatized to high ammonia levels (1.0 and 1.4 g NH_3-N L^{-1} for mesophilic and thermophilic conditions, respectively) with a batch cultivation method. In addition, another acclimatization (up to 10.0 g NH_4^+-N L^{-1}) performed in a continuously stirred tank reactor (CSTR) was also achieved. The microbial community composition changed significantly in both studies, and *Methanosarcina* spp. were found to be dominant in the final ammonia-tolerant methanogenic community, indicating their important role in overcoming the ammonia toxicity.

Moreover, focusing on the future full-scale application of bioaugmentation, a fast and efficient method to enrich the ammonia-tolerant consortia was developed based on the comparison of different acclimatization methods. The results demonstrated that the fed-batch was the most efficient method to acclimatize the ammonia-tolerant consortia. Specifically, compared to the batch acclimatization method, fed-batch saved up to 150% incubation time, achieved two times higher free ammonia (FAN) levels and improved 37%-153% methanogenic activity.

Thereafter, two bioaugmentation studies were performed to investigate the bioaugmentation efficiency and unravel the working mechanism. The results demonstrated a positive effect of bioaugmentation on improving the performance of the ammonia inhibited reactors. Firstly, after the

bioaugmentation of pure hydrogenotrophic *Methanoculleus bourgensis*, 28% methane production improvement was observed in a mesophilic CSTR reactor. Secondly, two bioaugmentation inocula were used under thermophilic conditions: an enriched ammonia-tolerant methanogenic consortium, and a mixed inoculum of a pure hydrogenotrophic methanogen and the enriched consortium. Compared to the control, 11-13% higher methane production was detected in the bioaugmented reactors with both inocula. In addition, compared to the bioaugmentation only with the enriched consortium, a faster methane recovery rate was observed by using the mixed inoculum. Based on the results, it was proposed that the instant hydrogen partial pressure reduction by the bioaugmented hydrogenotrophic methanogens played a significant role in alleviating ammonia inhibition in the AD process. Moreover, the "microbiological domino effect" was identified as the key mechanism of a successful bioaugmentation. In other words, even though the bioaugmented microorganisms were not the most abundant members in the microbiome, they were able to trigger an overall microbial community change towards to a more efficient AD microbiota.

Additionally, the impacts of other physicochemical factors, i.e. long chain fatty acid (LCFA) and different ammonia sources, on ammonia inhibition in the AD process were also assessed in this Ph.D. project. An ammonia-LCFA synergetic co-inhibition effect was identified in both batch and CSTR reactors. The results suggested that β-oxidation of LCFA was inhibited by high ammonia levels, thus resulted in the excess LCFA levels, which triggered the synergistic co-inhibition between ammonia and LCFA. Moreover, urea was found to be a stronger inhibitor compared to NH_4Cl. The higher toxicity of urea was attributed to the momentary higher FAN and pH levels during urea hydrolysis. Meanwhile, the results also demonstrated that the hydrogenotrophic methanogens were more robust than aceticlastic methanogens regardless of ammonia sources.

Overall, this study developed an efficient method to acquire bioaugmentation inocula and proved the effectiveness of bioaugmentation. Deep insight into the microbial community dynamics unravelled the bioaugmentation working mechanism and expanded the understanding of the ammonia-tolerant microbiota. The obtained findings suggested a practical solution for the future efficient utilization of nitrogen-rich substrates in the full-scale anaerobic digesters.

Dansk sammenfatning

Anaerob udrådning (AD) er en udbredt biologisk teknologi til udvinding af energi (i form af biometan, CH_4) fra forskellige bioaffald og biomasser. I de senere år er kvælstofrige substrater, såsom kyllingegødning, slagteriaffald, mikroalger osv. blevet attraktive AD-kilder på grund af deres høje metanpotentiale. Imidlertid hæmmer høje ammoniakniveauer forårsaget af nedbrydningen af disse nitrogenholdige substrater AD-processen, hvilket resulterer i akkumulering af flygtige fedtsyrer (VFA) og lav metanproduktion i forhold til metanpotentialet i disse substrater. I denne Ph.D. afhandling er der udviklet en effektiv metode til afhjælpning af ammoniakhæmning i AD-processen (bioaugmentering), og dermed til en forøgelse af metanproduktionen.

Først og fremmest er forudsætningen for en vellykket bioaugmentering at få fat i de ammoniak-tolerante metanogene konsortier og bruge dem som podemateriale for bioaugmentering en. I denne afhandling vurderedes mulighederne for at tilvænne mikrobielle konsortier til høje ammoniakniveauer. Resultaterne viste, at podematerialer taget fra to mesofile og tre termofile fuldskala biogasanlæg med succes blev tilvænnet høje ammoniakniveauer (1,0 og 1,4 g NH_3-N L^{-1} for henholdsvis mesofil og termofile betingelser) ved en batch dyrkningsmetode. Derudover blev en yderligere tilvænning (op til 10,0 g NH_4^+-N L^{-1}) opnået i en kontinuert omrørt reaktor (CSTR). Den mikrobielle sammensætning af bakteriekulturen ændrede sig betydeligt i begge undersøgelser og *Methanosarcina* spp. blev fundet dominerende i de resulterende ammoniaktolerante metanogene konsortier, hvilket angav deres vigtige rolle for at overvinde ammoniak hæmningen.

Desuden blev der udviklet en hurtig og effektiv metode til at opformere de ammoniaktolerante konsortier med udgangspunkt i sammenligningen af forskellige akklimatiseringsmetoder og med fokus på den fremtidige fuldskala anvendelse af bioaugmentering. Resultaterne viste, at fed-batch metoden var den mest effektive tilvænningsmetode for de ammoniak-tolerante konsortier. I en sammenligning med konventionel batch opnåede fed-batch metoden op til 150% reduktion i inkubationstiden, modstod to gange højere fri ammoniak (FAN) niveauer og forbedrede metanproduktion aktiviteten med 37% -153%.

Derefter blev der udført to bioaugmentering forsøgsrunder for at undersøge bioaugmenterings effektiviteten og for at opklare centrale mekanismer i effekten. Resultaterne viste en positiv virkning af bioaugmentering på forbedring af effektiviteten af ammoniak inhiberede reaktorer. I første forsøgsrunde

gav en bioaugmentering af en ren hydrogenotrof *Methanoculleus bourgensis* en 28% forøgelse af metan produktionen i en mesofil CSTR-reaktor. I anden forsøgsrunde blev to bioaugmenterings-podematerialer anvendt under termofile betingelser: et beriget ammoniak-tolerant metanogent konsortium og et blandet podemateriale af en ren hydrogenotrof methanogen og det berigede konsortium. Sammenlignet med kontrollen blev der målt 11-13% højere metanproduktion i de bioaugmenterede reaktorer med begge podematerialer. Derudover resulterede bioaugmenteringen med det blandede podemateriale i en hurtigere methanudvindingshastighed i forhold til det berigede konsortium alene. På baggrund af resultaterne blev det antaget, at den øjeblikkelige hydrogenpartialtryksreduktion af de bioaugmenterede hydrogenotrofe methanogener spillede en væsentlig rolle for at overvinde ammoniakinhiberingen af AD-processen. Desuden blev den "mikrobiologiske dominoeffekt" identificeret som den centrale mekanisme for en vellykket bioaugmentation. Med andre ord, selvom de bioaugmenterede mikroorganismer ikke var i høj koncentration i blandingen, kunne de udløse en overordnet ændring af den mikrobielle sammensætning hen imod en mere effektiv AD proces.

Endvidere blev virkningerne af andre fysisk-kemiske faktorer, f.eks. langkædede fedtsyrer (LCFA) og forskellige ammoniakkilder på ammoniakinhibering af AD-processen vurderet i denne Ph.D. afhandling. En ammoniak-LCFA-synergistisk hæmningseffekt blev identificeret i både batch- og CSTR-reaktorer. Resultaterne antyder, at β-oxidationen af LCFA blev hæmmet ved høje ammoniakniveauer, hvilket resulterede i overskydende LCFA-niveauer, som udløste den synergistiske inhibering mellem ammoniak og LCFA. Desuden blev urinstof som ammoniakkilde fundet at være en stærkere inhibitor sammenlignet med NH_4Cl. Den højere hæmning fra urinstof blev tilskrevet det øjeblikkeligt højere FAN- og pH-niveau ved urinstof hydrolyse. Samtidigt viste resultaterne også, at de hydrogenotrofe methanogener var mindre følsomme for hæmning end eddikesyre metanogener uanset ammoniakkilde.

Samlet set udvikledes fra disse forsøgsrækker en effektiv metode til at producere bioaugmenterings podematerialer og de beviste effektiviteten af bioaugmentering. Dyb indsigt i den mikrobielle dynamik opklarede bioaugmenteringsmekanismen og udvidede forståelsen af det ammoniak-tolerante mikrobielle samfund. De opnåede resultater angiver en praktisk løsning til den fremtidige mere effektive energiudnyttelse af nitrogenholdige substrater i fuldskala anaerobe anlæg.

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Abbreviations

AD	Anaerobic digestion
VFA	Volatile fatty acid
Mtoe	Million tonnes of oil equivalent
IHT	Interspecies hydrogen transfer
SAO	syntrophic acetate oxidation
SAOB	syntrophic acetate oxidizing bacteria
CSTR	Continuously stirred tank reactor
TAN	Total ammonia
FAN	Free ammonia
LCFA	Long chain fatty acid
HRT	Hydraulic retention time
OLR	Organic loading rate
EU	European Union
UASB	Up-flow anaerobic sludge blanket reactor
VSS	Volatile suspended solids
OTU	Operational taxonomic unit
BAN medium	Basal anaerobic medium

1 Introduction

1.1 Background

The development of human society relies on energy consumption. The global total energy consumption increased gradually in the past years, from 8560 million tonnes of oil equivalent (Mtoe) in 1990 to 13730 Mtoe in 2017 (Enerdata, 2018). However, the high proportion of fossil fuel in the total energy consumption (>80%) (IEA, 2015) resulted in huge greenhouse gases emissions and brought a lot of environmental issues, such as global warming, acid rain, air/water pollution, etc. Thus in order to solve these problems, many countries globally worked together and reached many agreements to decrease total fossil fuel consumption by increasing the share of renewable energies. For example, the European Union (EU) Commission sets targets that 20% and 32% final energy consumption will come from renewable sources by 2020 and 2030, respectively. Nevertheless, the latest global energy consumption report (Enerdata, 2018) showed that fossil fuel consumption increased in 2017, especially that coal consumption rebounded with 1% increase after a 3-year decline. Moreover, the CO_2 emissions also increased 2% compared to the year of 2016. Therefore, it is still urgent to explore more solutions to increase the share of renewable energies in the total energy consumption.

On the other hand, waste is an inevitable by-product during the development of society. In recent years, the amount of waste has increased significantly. For example, the production of municipal solid waste was 1.3 billion tonnes in 2012 and is expected to be 2.2 billion tonnes in 2025, which makes waste management be a big challenge (Hoornweg & Bhada-Tata, 2012). Recycling and reusing the waste is a crucial measure in order to live in a more sustainable society.

Therefore, from the view of energy saving and waste management, recovering energy from waste is becoming a worldwide topic (Kothari *et al.*, 2010). Considering the high organic matter content in the produced waste, anaerobic digestion (AD) plays an important role in this energy recovery process. Its advantage lies on that energy (in the form of biomethane, CH_4) is recovered by treating varieties of biowaste, such as agricultural and forestry waste, industrial waste and wastewater, municipal solid waste, sewage sludge, etc. (Kougias & Angelidaki, 2018). However, many inhibitors exist in this complex biological process, which cause process instability and poor utilization

of the waste. Among these inhibitors, ammonia is the most common one. It was reported that biogas plants operating under high ammonia levels usually lost more than 30% methane production potential (Fotidis *et al.*, 2014). Thus, it is of great importance to optimize AD process by overcoming ammonia inhibition and recover the residual energy.

1.2 AD process and microbiology

AD is a multistep biological process, which converts complex organic matters finally into methane (CH_4, 50-70%) and carbon dioxide (CO_2, 30-50%) in the absence of oxygen (Fig. 1). It happens in the natural environments, such as landfills, sediments, intestinal tracts of animal, etc. However, since two hundred years ago, human beings started using this technology in an artificial system (biogas reactor) to produce energy and treat biowaste. In general, AD is accepted to consist of four main steps, named hydrolysis, acidogenesis, acetogenesis and methanogenesis. Each step is finished with the participation of different microorganisms, mainly bacteria in the former three steps and archaea for the last step.

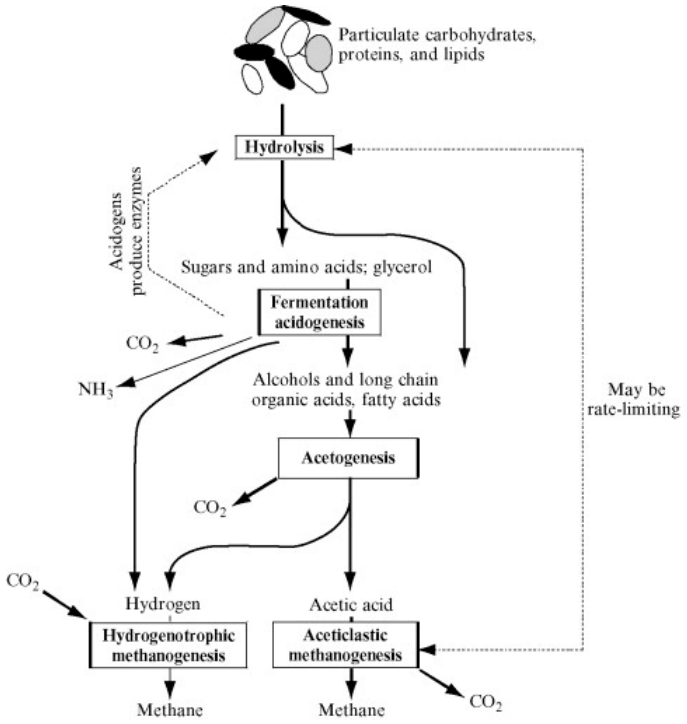

Figure 1. The Key steps of anaerobic digestion process [adapted from Angelidaki *et al.* (2011)]

1.2.1 Hydrolysis

The AD substrate is complex, mainly consists of different biopolymers, such as carbohydrate, protein, and lipid. These biopolymers are too big for the mi-

croorganisms to intake into cells. Thus during hydrolytic step, all the complex polymers are decomposed into soluble monomers by the extracellular hydrolytic enzymes. Specifically, carbohydrates are hydrolyzed into monosaccharides, while proteins into amino acids, and lipids into long chain fatty acids and glycerol (Angelidaki *et al.*, 2011). Hydrolysis is usually regarded as the rate-limiting step of the overall AD process (Appels *et al.*, 2008), especially when complex biofibers (a mixture of cellulose, hemicellulose, and lignins) are the main substrates.

During this step, the extracellular hydrolytic enzymes, such as cellulose, protease, lipase, etc., produced by hydrolytic bacteria, are the most crucial factors. Previous studies showed that phylum *Bacteroidetes* and *Firmic*utes includes most of the hydrolytic bacteria (Ali Shah *et al.*, 2014, Xia *et al.*, 2014), such as genera *Anaerocellum* and *Clostridium*. Moreover, compared to methanogens, these hydrolytic bacteria are less sensitive to environmental changes, such as pH and temperature disturbance.

1.2.2 Acidogenesis

The products from hydrolysis are converted into mainly volatile fatty acids (VFAs) in acidogenesis step, including formic, acetic, propionic, butyric, etc., acids. For example, the monosaccharides are fermented into C3 products (lactate and propionate) through Emben-Meyerhof-Parnas or Entner Doudoroff pathway (Angelidaki *et al.*, 2011). Meanwhile, some other products are also produced, such as hydrogen, carbon dioxide, alcohols (methanol and ethanol), etc. A variety of species belonging to *Clostridia* and *Bacteroidales* are found to be responsible for this step (Svensson *et al.*, 2007, Angelidaki *et al.*, 2011, Traversi *et al.*, 2012). Most of the acidogens are strict anaerobes, but facultative anaerobes are also found, such as *Streptococci* and *Enterobacteriaceae*, to use oxygen and tolerant the fermentation environment changes (Ali Shah *et al.*, 2014).

It must be mentioned that ammonia is also produced during this step from amino acids degradation. Even though a proper ammonia concentration (<200mg NH_4^+-N L^{-1}) is needed as nutrient for the microorganism growth, high ammonia levels can result in inhibition on the AD process.

1.2.3 Acetogenesis

VFAs (except for formic and acetic acid) and ethanol from acidogenesis step cannot be directly used by methanogens, which need to be further degraded into acetate, H_2 and CO_2. The process for acetate synthesis is so called aceto-

genesis, which mainly consists of two pathways: 1) degradation of other VFAs, and 2) reduction of CO_2. *Syntrophomonas* and *Syntrophobacter* are two major genera found to be responsible for acetogenesis (Ali Shah *et al.*, 2014). However, as shown in Table 1, most of the acetogenic reactions are thermodynamically unfavourable, unless the produced H_2 is consumed immediately. Therefore, a syntrophic relationship between the H_2 producer and consumer is formed, named as interspecies hydrogen transfer (IHT). IHT plays an important role in this step to keep a low hydrogen partial pressure, which creates thermodynamically favourable conditions for acetogenesis of other VFAs.

The acetate formation from the reduction of CO_2 is also called homoacetogenesis, which is performed via Wood-Ljungdahl pathway by hydrogen-utilizing acetogens (homoacetogens). In this scenario, CO_2 is used as electron acceptor and H_2 is used as electron donor. The typical homoacetogens can be found in *Clostridiales* order, such as *Acetobacterium woodii* and *Clostridium aceticum* (Diekert & Wohlfarth, 1994).

Table 1. Standard Gibbs free energy changes of some reactions involved in acetogenesis and methanogenesis step [Adapted from Angelidaki *et al.* (2011)]

Reaction	$\Delta G_o'$ (kJ reaction^{-1})
VFAs oxidation	
$CH_3COO^- + 4\ H_2O \rightarrow H_2 + 2\ HCO_3^- + H^+$	+105
$CH_3CH_2COO^- + 3\ H_2O \rightarrow CH_3COO^- + HCO_3^- + H^+ + 3\ H_2$	+76
$CH_3CH_2CH_2COO^- + 2\ H_2O \rightarrow 2\ CH_3COO^- + H^+ + 2\ H_2$	+48
Homoacetogenesis	
$4\ H_2 + 2\ HCO_3^- + H^+ \rightarrow CH_3COO^- + 4\ H_2O$	-105
Methanogenesis	
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31
$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O$	-136

1.2.4 Methanogenesis

Methanogenesis is the last step of AD process. It is generally accepted as the most sensitive step to environmental changes and toxicants. Three pathways are identified for methane formation: aceticlastic pathway, in which acetate is directly cleaved into CH_4 and CO_2; hydrogenotrophic pathway, in which H_2 and CO_2 react together to form CH_4; and methylotrophic pathway, which means the methylated C1 compounds, such as methanol and methylamine, is converted to CH_4. Acetate is the most important precursor to produce methane, which contributes to approximately 70% of the total methane produc-

tion. However, except for being directly used via aceticlastic pathway, acetate can also be used through hydrogenotrophic pathway. In this scenario, acetate is first converted into H_2 and CO_2 through syntrophic acetate oxidation (SAO), and then H_2 and CO_2 were consumed by hydrogenotrophic methanogens. The bacteria that oxidize acetate into H_2 and CO_2 are called syntrophic acetate oxidizing bacteria (SAOB). Up to date, only five SAOB species (three mesophilic and two thermophilic types) are isolated: *Clostridium ultunense* (Schnürer, 1996), *Syntrophaceticus schinkii* (Westerholm *et al.*, 2010), *Tepidanaerobacter acetatoxydans* (Westerholm *et al.*, 2011), *Thermacetogenium phaeum (Hattori et al., 2000)* and *Pseudothermotoga lettingae* (Balk *et al.*, 2002). However, some other genera without isolation are also proposed to be SAOB, such as genera belong to species *Coprothermobacter*, orders *Bacteroidales*, *Clostridiales* and *Thermoanaerobacterales*, and phylum *Thermotogae* (Nobu *et al.*, 2015, Ho *et al.*, 2016, Müller *et al.*, 2016, Campanaro *et al.*, 2018).

Unlike the previous steps (hydrolysis, acidogenesis and acetogenesis) mediated by bacteria, methanogenesis is performed by archaea (methanogens). Up to data, seven different orders are reported to perform methanogenesis: *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales*, *Methanobacteriales*, *Methanococcales*, *Methanopyrales* and *Methanomassiliicoccales* (Angelidaki *et al.*, 2011, Borrel *et al.*, 2014). Most of these orders are hydrogenotrophic methanogens, and only genera *Methanosaeta* and *Methanosarcina* perform aceticlastic pathway. Moreover, *Methanosarcina* is the only genus that has the ability to perform both aceticlastic and hydrogenotrophic pathways (De Vrieze *et al.*, 2012).

1.3 Ammonia inhibition in the AD process

AD consists of different biological reactions, in which a variety of microorganisms are involved. Thus a lot of inhibitors exist inhibiting the activities of these microorganisms (Chen *et al.*, 2014), such as ammonia, sulphide, light/ heavy metals, organics (e.g. chlorophenols), etc. Among them, ammonia, which is produced during the degradation of proteins, urea and nucleic acids, is the most common one. Total ammonia levels (TAN) in the reactor equals to the sum of the ammonium ions (NH_4^+) and free ammonia (FAN, NH_3). Moreover, FAN, which increases along with pH and temperature (Eq. (1)), is identified as the most toxic form (Massé *et al.*, 2014).

$$\mathrm{FAN} = \frac{\mathrm{TAN}}{1+\frac{10^{-\mathrm{pH}}}{K_a}} \qquad \text{Eq. (1)}$$

Where K_a is the dissociation constant determined by temperature and it equals to 1.29×10^{-9} at mesophilic condition (37±1°C) and 3.91×10^{-9} at thermophilic condition (55±1°C).

1.3.1 Ammonia inhibition mechanism

Among the four steps of AD process, methanogenesis was proved to be the most sensitive step to ammonia (Yenigün & Demirel, 2013). However, hydrolytic and acidogenic efficiency were also influenced by high ammonia levels (>6.5 g NH_4^+-N L^{-1}) (Lü *et al.*, 2008, Niu *et al.*, 2013). Different theories about the inhibition mechanism were also proposed as follows (Wittmann *et al.*, 1995, Gallert *et al.*, 1998):

- Methane formation enzyme may be directly inhibited by ammonium ion;
- FAN may diffuse into microorganisms' cell and become ammonium ion, thus causes intracellular pH change;
- FAN diffusion into cells might cause intracellular potassium deficiency. Moreover, the process excluding potassium out of cell also consumes extra energy.

However, the diffusion of FAN into the cell depends on the physiology of the microbes. Thus different microbes have different response/ tolerance to the same ammonia levels. For example, the hydrogenotrophic methanogens are often reported to have higher ammonia tolerance compared to aceticlastic methanogens (Karakashev *et al.*, 2005, Song *et al.*, 2010).

1.3.2 Reactor performance under ammonia inhibition

Ammonia inhibition in different reactor types with different substrates was extensively studied in the past. It was reviewed by Chen *et al.* (2008) that server ammonia inhibition was observed when ammonia levels ranged from 1.7 to 14.0 g NH_4^+-N L^{-1} depending on different digestion environment. For example, in mesophilic continuously stirred tank reactors (CSTR) digesting with egg albumin powder, 50% methane production loss was reported when ammonia levels increased from 0.8 to 5.5 g NH_4^+-N L^{-1}, together with a high VFA accumulation up to 18.0 g L^{-1} (Westerholm *et al.*, 2011). In another study treating with fishery sludge, server inhibition was detected at ammonia levels ranging from 6.4 to 7.5 g NH_4^+-N L^{-1} (Gebauer & Eikebrokk, 2006). Furthermore, it was reported that the thermophilic AD process usually suffers more severe inhibition compared to mesophilic conditions. This is because of the higher FAN levels created by the higher temperature. For example, thermophilic degradation of cattle manure was inhibited at ammonia levels of 4.0

g NH_4^+-N L^{-1} (Angelidaki & Ahring, 1993). Moreover, a thermophilic reactor fed with a mixture of paper, food waste and yard waste, lost its 50% and 100% methane production even at 1.5 and 2.5 g NH_4^+-N L^{-1}, respectively (Kayhanian, 1994).

1.3.3 Strategies to alleviate ammonia inhibition

In order to recovery the lost methane, different methods aiming to overcome ammonia inhibition were tested in the past years. Firstly, decreasing pH and/ or temperature levels of the reactor to have a low FAN levels were proved to increase methane production (Zeeman *et al.*, 1985, Angelidaki & Ahring, 1994). Secondly, addition of absorbing materials, such as zeolite, to trap ammonia thus reduce the TAN levels, were also a potential way to increase methane yield (Hansen *et al.*, 1999, Kougias *et al.*, 2013). Moreover, dilution the reactor content with either tap water or digested manure was investigated in batch and lab-scale continuous reactors. The result showed a faster methane recovery rate in reactors with dilution compared to the reactor without any changes (Nielsen & Angelidaki, 2008). Fourthly, co-digestion of the protein-rich substrate and high carbon content substrate to achieve a suitable C/N ratio was also successfully used to recovery methane production (Tian *et al.*, 2015, Tsapekos *et al.*, 2017). Additionally, some others measures were also taken trying to remove the ammonia from the reactor, such as air stripping and microbial electrochemical cell (Zhang & Angelidaki, 2015, Yuan *et al.*, 2016). The results turned out to be effective supported by the improved methane production.

However, almost all the aforementioned solutions either are cost-expensive or create other problems, such as high waste volume. Besides, some of the solutions also need to change the infrastructure of the original reactor setup. All the mentioned factors limit the applicability of these solutions (Fotidis *et al.*, 2014). Some recent studies based on microbial management technology, i.e. bioaugmentation, successfully increased the methane production and decreased the VFA levels (Fotidis *et al.*, 2014, Fotidis *et al.*, 2017, Li *et al.*, 2017). The bioaugmentation method is easy to operate without the need to change any infrastructures. However, there are still some challenges from lab-scale findings to full-scale application, such as the efficient enrichment and cultivation method for the ammonia-tolerant methanogenic consortia.

1.4 Bioaugmentation application in AD field

Bioaugmentation is a microbial management technology, which introduces active biomass (enriched consortium and/ or pure strain) with specific func-

tions into a biological system to achieve some specific purposes (Stephenson & Stephenson, 1992). This technology has been used successfully in many fields, such as aerobic wastewater treatment (Ivanov *et al.*, 2006), soil and groundwater bioremediation (Vogel, 1996), and hazardous waste control (Schauer-Gimenez *et al.*, 2010). In recent years, bioaugmentation has also been used in AD field to solve some problems. Different pure strains, such as *Acetobacteroides hydrogenigenes*, *Clostridium thermocellum*, and *Meliori-bacter roseus*, were used to improve methane production from lignocellulosic substrates (Zhang *et al.*, 2015, Tsapekos *et al.*, 2017). As a result, up to 34% higher methane yield was achieved in the bioaugmented reactors compared to the control. Moreover, bioaugmentation also showed the ability to overcome problems from overloading and oxygen exposure (Schauer-Gimenez *et al.*, 2010, Tale *et al.*, 2011, Tale *et al.*, 2015). Improved methane production from microalgae and LCFA were also observed by using different bioaugmentation strains, such as *Clostridium thermocellum* and *Clostridium lundense* (Cirne *et al.*, 2006, Fan *et al.*, 2013). Additionally, bioaugmentation to increase methane production from protein-rich substrates were also reported. For example, proteolytic bacteria (*Bacillus coagulans*, *Bacillus subtilis* and *Pseudomonas fluorescens*) increased the hydrolysis of protein (Kovacs *et al.*, 2015), and *Methanoculleus* spp. recovered methanogenic activity under high ammonia levels (Fotidis *et al.*, 2014, Fotidis *et al.*, 2017).

However, challenges still exist even with some encouraging results, which prevent the further scale up of bioaugmentation technology. First of all, it is not easy to choose the right bioaugmentation culture. The culture needs to grow fast enough to avoid being washed out (Westerholm *et al.*, 2012), and needs to outcompete the indigenous microorganisms to thrive in the new system (Nkemka *et al.*, 2015). Moreover, a fast and cheap cultivation method for the bioaugmentation culture needs to be developed. Additionally, the storage and transportation of the bioaugmentation culture is still an unsolved problem when it comes to full-scale application. Last but not least, critical biomass, which means the minimum amount of biomass for a successful bioaugmentation, is also identified as a bottleneck to make this technology economically feasible (Fotidis *et al.*, 2014).

1.5 Objectives and thesis structure

1.5.1 Objectives

The aim of this Ph.D. project is to develop an innovative and reliable bioaugmentation method to alleviate ammonia inhibition in AD process and es-

tablish an ammonia-tolerant community in anaerobic digesters. In this way, methane production efficiency will be enhanced for the full-scale biogas reactors suffering from ammonia toxicity. In particular, this study has three main objectives: the first objective is to have a clear understanding of the enriched ammonia-tolerant methanogenic consortia; the second one is to recover methane production from ammonia inhibited continuous reactors through different bioaugmentation strategies; the third one is to understand the impact of other factors during AD process on ammonia inhibition study.

Specifically, the detailed objectives of the whole project are:

- Acclimatize the normal methanogenic consortia to high ammonia levels (>1.0 g NH_3-N L^{-1}) at both mesophilic and thermophilic conditions; (Paper I)
- Have a deep insight into microbial community of the enriched consortia and identify the dominant acetate methanogenic pathway; (Paper I)
- Develop an efficient acclimatization method to acquire ammonia-tolerant methanogenic consortia; (Paper II)
- Acclimatize methanogenic consortium to high ammonia levels (>7.0 g NH_4^+-N L^{-1}) in continuous reactor; (Paper III)
- Reveal the microbial dynamics during the acclimatization process. (Paper III)
- Achieve successful bioaugmentation in CSTR reactor fed with natural protein-rich 3rd generation AD substrate operated at extremely high ammonia levels (11.0 g NH_4^+-N L^{-1}); (Paper IV)
- Develop effective bioaugmentation strategies for thermophilic continuous reactors; (Paper V)
- Depict the microbial community dynamics before and after bioaugmentation; (Paper IV& Paper V)
- Propose a basic bioaugmentation working mechanism. (Paper IV& Paper V)
- Identify the ammonia and LCFA synergetic co-inhibition effect during AD process; (Paper VI)
- Propose a potential mechanism to explain the ammonia and LCFA synergism; (Paper VI)

- Understand the difference of ammonia inhibition on pure methanogens from two ammonia sources (i.e. urea and ammonium chloride). (Paper VII)

1.5.2 Structure of the thesis

In Chapter 2, the microbial characterization of ammonia-tolerant methanogenic consortia is presented, together with the dominant acetate methanogenic pathway. Moreover, advantages and disadvantages of different acclimatization methods are compared. Finally, acclimatization of methanogenic culture to extremely high ammonia levels in CSTR reactors fed with mainly 3rd generation AD substrate is discussed.

In Chapter 3, two different bioaugmentation studies (mesophilic and thermophilic conditions) are described. Microbial community changes before and after bioaugmentation are presented. Moreover, a proposed bioaugmentation working mechanism is also discussed in this chapter.

In Chapter 4, the ammonia and LCFA synergetic co-inhibition effect is discussed, together with a proposed mechanism for this synergism. Additionally, ammonia inhibition from different ammonia sources on pure methanogenic strains is also described in this chapter.

In Chapter 5 and Chapter 6, conclusions of the whole Ph.D. project and the future perspectives were illustrated, respectively.

2 Acclimatization and characterization of enriched ammonia-tolerant methanogenic consortia

In recent years, more and more protein-rich substrates, such as chicken manure, slaughterhouse waste, microalgae, etc., are used as AD feedstock (Angelidaki *et al.*, 2011, Mahdy *et al.*, 2017). However, high ammonia levels are usually formed during the degradation of protein-rich substrates, which inhibit the AD process (Chen *et al.*, 2008). As discussed in chapter 1.3.3, different methods are proposed to overcome ammonia problem, and bioaugmentation seems to be the most promising one. Therefore, an efficient bioaugmentation inoculum is a prerequisite determining the bioaugmentation effect. However, the understanding of the ammonia-tolerant methanogenic consortia is still scarce. For example, controversial observations were reported about the microbial community composition of the consortia. Some researchers claimed the important role of hydrogenotrophic methanogens at high ammonia levels (>3.0 g NH_4^+-N L^{-1}) (Sun *et al.*, 2014, Müller *et al.*, 2016); while others also found the high abundance of aceticlastic *Methanosarcina* spp. in reactors with high ammonia concentration (Calli *et al.*, 2005, Fotidis *et al.*, 2013). Considering the contradictions, microbial characterization of the potential bioaugmentation inocula, i.e. the ammonia-tolerant methanogenic consortia, need further investigation.

Moreover, even though some successful bioaugmentation results were obtained before (Fotidis *et al.*, 2014, Fotidis *et al.*, 2017, Li *et al.*, 2017), the availability of the ammonia-tolerant consortia is always identified as one of the main bottlenecks that determine the scale up of bioaugmentation. The acclimatization process is usually time-consuming (Rajagopal *et al.*, 2013). An efficient acclimatization and cultivation method has not been reported before. In order to apply bioaugmentation into the full-scale biogas plants, it is inevitable to develop a fast method to prepare a large amount of the bioaugmentation inoculum in a timeframe as short as possible. Therefore, three different experiments were conducted in this chapter, to study the characteristics of the ammonia-tolerant consortia, and to develop the efficient acclimatization method for the ammonia-tolerant consortia.

2.1 Characterization of the ammonia-tolerant methanogenic consortia

In paper I, acclimatization of five different inocula (two mesophilic: M1 and M2; three thermophilic: T1, T2 and T3) to high ammonia levels were performed in batch reactors (Table 2). The five inocula were initially taken from different Danish full-scale biogas plants. During the acclimatization process, basal anaerobic medium (BAN medium) (Angelidaki *et al.*, 1990) was used as the cultivation medium, and acetate as the main carbon source. Additionally, yeast extract was also added to offer necessary nutrients. Each enrichment step was ceased only when the methane production was close to the maximum expected value.

Table 2. Experimental setup of the stepwise enrichment process [Adapted from **paper I**]

Enrichment steps	Mesophilic inocula			Thermophilic inocula		
	TAN	pH	FAN	TAN	pH	FAN
	g NH_4^+-N L^{-1}		mg NH_3-N L^{-1}	g NH_4^+-N L^{-1}		mg NH_3-N L^{-1}
1	3.0	7.0	38.2	3.0	7.0	112.9
2	4.0	7.0	50.9	4.0	7.0	150.5
3	5.0	7.0	63.7	5.0	7.0	188.1
4	6.0	7.0	76.4	6.0	7.0	225.8
5	7.0	7.0	89.1	7.0	7.0	263.4
6	7.0	8.0	799.8	5.0	8.0	1405.5
7	9.0	8.0	1028.3	-	-	-

2.1.1 Maximum growth rate (μ_{max})

On the one hand, the results showed that the μ_{max} at each enrichment step decreased during the acclimatization process. This is expected since toxicity increases with ammonia concentrations (Borja *et al.*, 1996, Yenigün & Demirel, 2013). On the other hand, even with a decreased μ_{max}, the enriched ammonia-tolerant consortia at the final step still catabolized acetate efficiently with a μ_{max} between 0.17 to 0.31 d^{-1}. It seems that these consortia can be potentially used as bioaugmentation culture in CSTR reactors without being washed out, because they had a much faster doubling time (2-3 days) compared to the normal HRTs (13-25 days).

2.1.2 Dominant methanogenic pathway

Dominant acetate methanogenic pathway (either aceticlastic or hydrogenotrophic) can be identified through radioisotopic analyses using labelled [2-

^{14}C] sodium acetate (Schnürer & Nordberg, 2008, Fotidis *et al.*, 2013). The current results showed that all the five ammonia-tolerant consortia were mainly performing aceticlastic methanogenic pathway. This agreed with Hao *et al.* (2015) reporting that acetate was catabolized to methane mainly through aceticlastic pathway independently of ammonia levels according to stable carbon (1, 2- ^{13}C) isotopic analysis.

2.1.3 Microbial community composition

16s rRNA gene sequencing was used to look into the microbial composition of the final ammonia-tolerant methanogenic consortia. The results showed a big difference between mesophilic and thermophilic consortia.

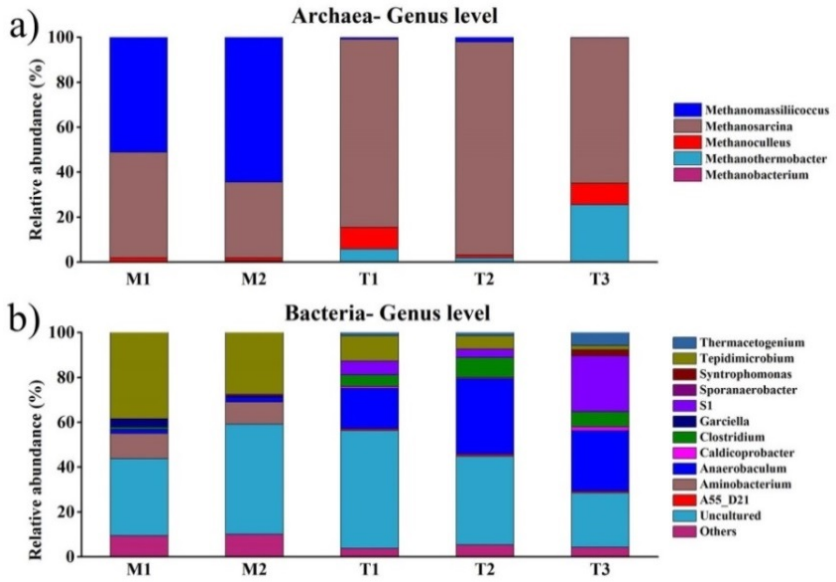

Figure 2. Taxonomic classification at genus levels of a) archaea and b) bacteria of the ammonia-tolerant consortia. Genera with relative abundance less than 0.5% were classified as "Others". [Adapted from **paper I**]

At mesophilic conditions, the community consisted of archaea and bacteria, with relative abundance of around 30% and 70%, respectively. *Methanomassiliicoccus* spp. and *Methanosarcina* spp. were the two most abundant genera (Fig. 2). Specifically, two OTUs that belong to *Methanomassiliicoccus* spp. were identified 96-98% similar to *Methanomassiliicoccus luminyensis* (Fig. 3). *M. luminyensis* is a newly reported strict methylotrophic methanogen (Popp *et al.*, 2016, Lin *et al.*, 2017). Thus it was a surprise to detect its dominance in the current study since 1) acetate, instead of methylated compounds, was used as the main carbon source, and 2) aceticlastic methanogenesis was the dominant acetate methanogenic pathway. Different possible explanations were proposed, such as the substrates for *M. luminyensis* might be produced

and acetate might be used directly by *M. luminyensis*. Unfortunately, due to the limitation of 16s rRNA sequencing technology, only taxonomic information was provided, rather than the genomic characteristics and the functional annotation. Therefore, to clarify the exact metabolic pathway of *M. luminyensis*, further investigations based on more advanced method are needed, such as total random gene sequencing and/ or metatranscriptomic technology. The other abundant methanogen was aceticlastic *Methanosarcina soligelidi*, which agreed with the dominance of *Methanosarcina* spp. at high ammonia levels in previous study (Calli *et al.*, 2005). Moreover, it also matched the radioisotopic results that aceticlastic pathway was the dominant methanogenic pathway.

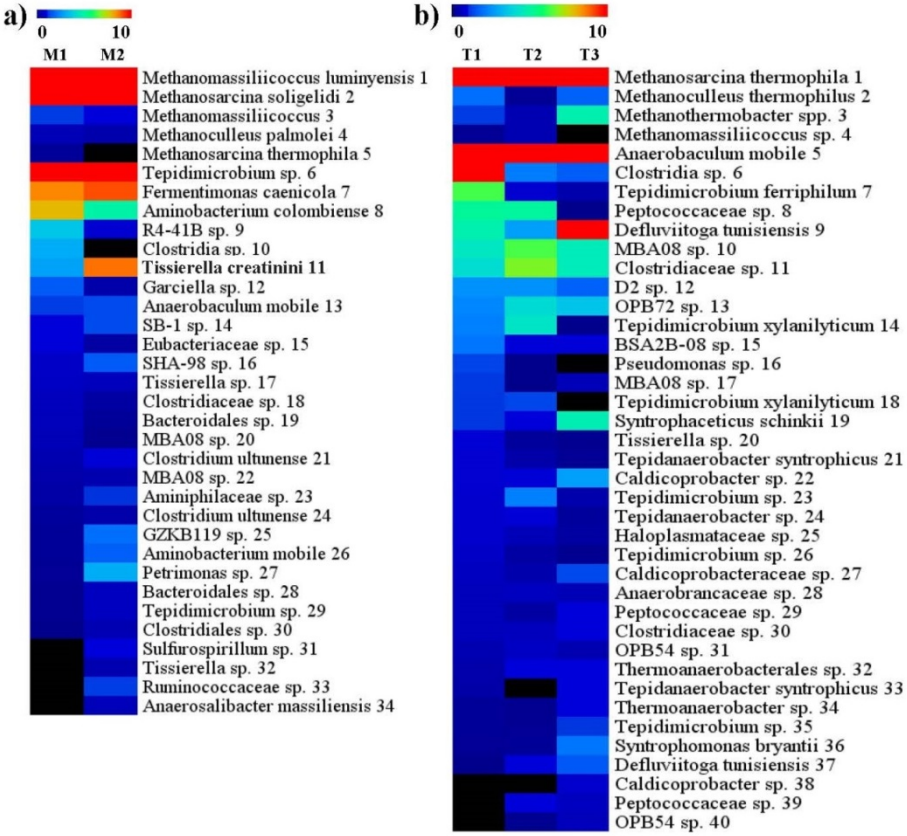

Figure 3. Relative abundance (with respect to the whole community) of interesting OTUs of a) mesophilic and b) thermophilic ammonia-tolerant consortia. [Adapted from **paper I**]

At thermophilic conditions, the relative abundance of archaea varied between 19-24%, and the rest were bacteria. The most dominant archaea was *Methanosarcina thermophila*, with relative abundance between 65-95% with respect to archaea community. *M. thermophila* is a well-known aceticlastic methanogenic species (Zinder *et al.*, 1985). Its high relative abundance in all the ammonia-tolerant consortia coincided with the dominant aceticlastic

methanogenic pathway according to radioisotopic analyses. Additionally, another two abundant methanogens were hydrogenotrophic *Methanoculleus thermophilus* and *Methanothermobacter thermautotrophicus*, indicating a certain degree of hydrogenotrophic methanogenesis in the consortia.

2.2 The efficient acclimatization method of ammonia-tolerant methanogenic consortia

2.2.1 Comparison between different reactor types

An efficient acclimatization method of ammonia-tolerant methanogenic consortia is needed for the scale up of bioaugmentation technology. In the previous studies, batch reactor was used to acclimatize and cultivate the ammonia-tolerant consortia (Westerholm *et al.*, 2012, Fotidis *et al.*, 2013, Fotidis *et al.*, 2014). However, its efficiency (in terms of incubation time, TAN and FAN levels achieved, methanogenic activity, etc.) has never been assessed and compared with other reactor types, such as fed-batch and continuous reactor.

Therefore, in Paper II, three cultivation methods (batch, fed-batch and CSTR) were compared in order to identify the most efficient method for ammonia-tolerant methanogenic consortia. Additionally, the different reactor performances of direct-exposure and stepwise-exposure to toxicity in batch reactor were reported (Fotidis *et al.*, 2013). Thus the two different batch assays (i.e. direct and stepwise exposure) were also performed in this study. The initial inocula were taken from two Danish full-scale biogas plants with TAN levels of 3.56 and 3.32 g NH_4^+-N L^{-1} for mesophilic and thermophilic conditions, respectively. The TAN levels were increased gradually by 1.0 g NH_4^+-N L^{-1} each time for stepwise-exposure batch, fed-batch and CSTR reactor type. Regarding the direct-exposure, the TAN levels were increased to the target levels directly. BAN medium and acetate were used as cultivation medium and main carbon source, respectively.

As shown in table 3, the results clearly showed that fed-batch was the best choice to acclimatize ammonia-tolerant consortia compared to batch and CSTR reactor type. Fed-batch method achieved the highest FAN levels among all the methods. Moreover, high methane yield and high methanogenic activity were also obtained within a short timeframe. For example, under thermophilic conditions, more than 40% shorter acclimatization time was achieved in fed-batch reactor compared to batch method, together with higher methane production and methanogenic activity. The possible explanations for the success of fed-batch lie on 1) the relatively stable microbial growth rate

controlled by the exponential feeding, and 2) the complete initial microbial community because of no effluent removal.

On the one hand, batch method took a longer time and reached lower FAN levels and lower methanogenic activity compared to fed-batch. On the other hand, low methane production with high VFA accumulation was observed in CSTR reactors even with only 1.0 g NH_4^+-N L^{-1} increase. It indicated that CSTR reactor was not efficient enough to acclimatize ammonia-tolerant consortia. This might be due to the washout effect of ammonia-tolerant methanogens (Fynn & Whitmore, 1984). However, it must be mentioned that different performance might occur with a smaller TAN increase (e.g. 0.5 g NH_4^+-N L^{-1}) each time and a longer HRT compared to the ones adopted in this study.

Additionally, the results also showed that hydrogenotrophic methanogens were more active than aceticlastic methanogens at high FAN levels (>540 mg NH_3-N L^{-1}); while it was opposite at low FAN levels (<210 mg NH_3-N L^{-1}). This observation agreed with Schnürer & Nordberg (2008) declaring that methanogenic pathway was shifted from aceticlastic to hydrogenotrophic during elevated ammonia levels.

Table 3. Comprehensive comparison between the three different acclimatization methods [Adapted from **paper II**]

		Highest TAN	Highest FAN	Incubation time	Methanogenic activity	Production efficiency
		g NH_4^+-N L^{-1}	mg NH_3-N L^{-1}	d	mmol CH_4 L^{-1} d^{-1}	%
Mesophilic inoculum	**Batch direct**	7.56	208	78	7.04	100
	Batch stepwise	7.56	181	125	10.13	100
	Fed-batch	6.56	549	84	16.75	86.5
	CSTR	4.56	490	-	20.21	32
Thermophilic inoculum	**Batch direct**	6.32	614	91	11.33	100
	Batch stepwise	6.32	542	158	20.99	100
	Fed-batch	6.32	1633	64	28.68	83.9
	CSTR	4.32	1425	-	27.43	30

2.2.2 Successful acclimatization in CSTR reactor

As shown in the previous section (paper II) that acclimatization of ammonia-tolerant consortia with CSTR method did not succeed. The main reason was

attributed to the washout effect of the methanogens. However, as stated before, a smaller TAN increase each time might have a different result due to the milder ammonia stress. Moreover, another possibility that resulted in the washout of microbes could be the simplicity of the BAN medium used in previous study. In another word, BAN medium does not contain any particles for the microbes to adhere to. On the one hand, it was reported that some support matrixes in continuous reactors could avoid the washout effect, such as carbon fiber textiles and carbon felt (Sawayama *et al.*, 2004, Sasaki *et al.*, 2011). On the other hand, cattle manure contains a lot of lignocellulosic fiber that may perform the same function as support matrix. Recently, microalgae are becoming an attractive AD substrate because they have a high methane potential and they do not competitive with food supply (Mahdy *et al.*, 2017). Microalgae, belonging to 3rd generation AD biomass, is also a natural protein-rich substrate (>50% protein in the dry mass).

Therefore, in this study (paper III), two CSTR reactors (as duplicate) fed with a mixture of cattle manure and microalgae (20/80, based on VS) were used to acclimatize methanogenic consortium to high ammonia levels. The TAN levels was increased from 3.3 to 10.0 g NH_4^+-N L^{-1} with a stepwise increase strategy of 0.5 g NH_4^+-N L^{-1} in each step. The experimental period was divided into four different phases, and the detailed operational parameters are shown in table 4.

Table 4. Operational parameters in different experimental phases [Adapted from **paper III**]

	Phase 1 (P1)	**Phase 2 (P2)**	**Phase 3 (P3)**	**Phase 4 (P4)**
Days	0-28	29-51	52-98	99-133
Substrate	20% cattle slurry + 80% microalgae (based on VS)			
OLR (g VS L^{-1} d^{-1})	1.95 ± 0.10			
TAN (g NH_4^+-N L^{-1})	3.3-3.8	4.3-6.0	6.5-8.0	8.5-10.0

The results, for the first time, demonstrated a successful acclimatization process up to 10 g NH_4^+-N L^{-1} in continuous reactors fed with natural protein-rich substrate. Specifically, as shown in Fig. 4, the methane production was stable with a small fluctuation throughout the whole 133 days experimental period. Moreover, the VFA levels in both reactors were always below 2400 mg L^{-1} and one of the two reactors was even below 1000 mg L^{-1}. The results agreed with previous studies reporting that methane production was still efficient when VFA levels were below 3000 mg L^{-1} (Ahring *et al.*, 1995, Siegert & Banks, 2005). Meanwhile, the pH levels of the two reactors fluctuated be-

tween 8.4 and 7.7 throughout the experiment, which were always within the optimal pH range (6.5-8.5) for AD process (Lay *et al.*, 1998).

16S rRNA gene sequencing results showed a clear microbial dynamics during the acclimatization process. Moreover, Chao 1 bias-corrected index decreased along with the increase of ammonia levels, indicating that not all the microbes in the original community can survive at high ammonia levels.

According to the sequencing results, the most abundant OTU was found to belong to *Shinella* sp., with an average relative abundance of 22% in all the microbial samples. The abundance of *Shinella* sp. didn't show any significant changes ($p>0.05$) under different ammonia levels. It was proposed by Sanz *et al.* (2017) that *Shinella* sp. played an important role in degrading polysaccharides and glycoprotein matrix of microalgae. Thus the constantly abundant *Shinella* sp. in the reactor indicated an efficient utilization of the substrates. Another noteworthy observation was that the SAOB *C. ultunense* (Schnürer, 1996) increased its abundance up to 60 times during the acclimatization process. Together with an increasing trend of hydrogenotrophic *Methanoculleus palmolei*, the increased SAOB indicated a degree of hydrogenotrophic methanogenesis at high ammonia levels.

Within the archaea community, the interesting observation was that the most dominant *Methanobrevibacter* sp. (relative abundance>60%) at low ammonia levels was replaced by *Methanosarcina* sp. (around 90%) at high ammonia levels. Meanwhile, *Methanoculleus palmolei* (5-8%) also emerged and increased its abundance along with the increase of TAN levels. This result was a surprise because hydrogenotrophic methanogens were usually found abundant in continuous reactors operated with high ammonia levels (Angenent *et al.*, 2002, Fotidis *et al.*, 2014). A possible explanation was that *Methanosarcina* spp. was reported to cluster together forming aggregates at high ammonia levels thus increasing the volume/ surface ratio to tolerant ammonia toxicity (Calli *et al.*, 2005, Goberna *et al.*, 2010). Another possibility was that *Methanosarcina* sp. may use hydrogen and carbon dioxide to produce methane. This was supported by the findings that *Methanosarcina soligelidi*, *Methanosarcina barkeri* and *Methanosarcina mazei* could perform hydrogenotrophic pathway (Liu *et al.*, 1985, Wagner *et al.*, 2013).

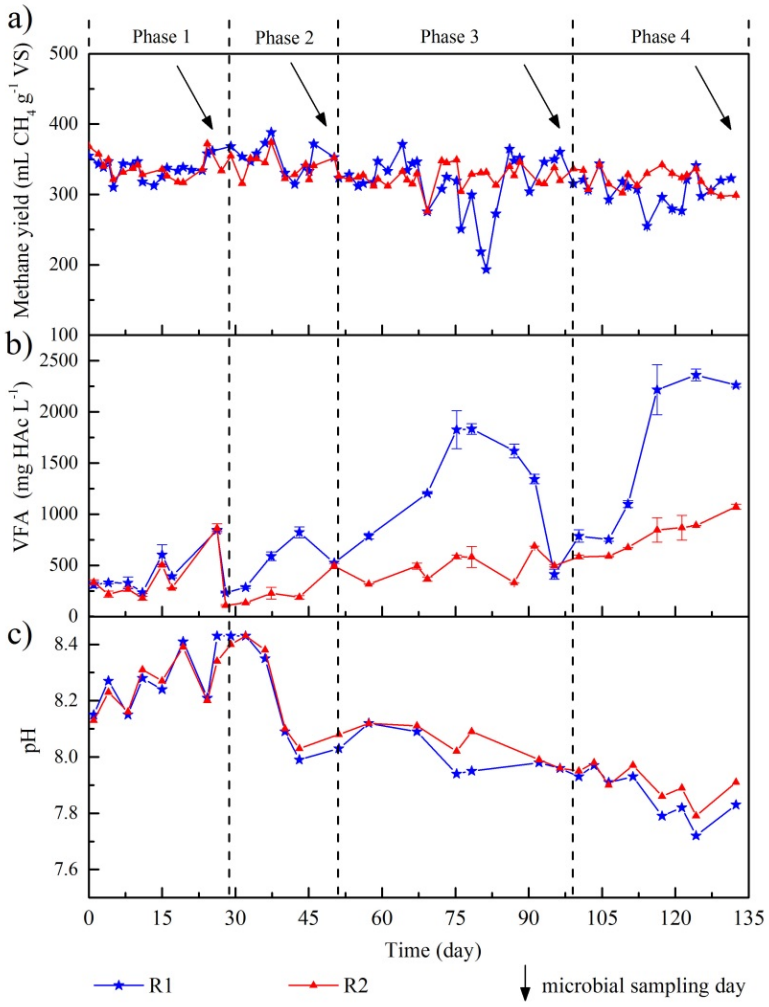

Figure 4. The performance parameters of the reactors a) methane yield, b) VFA and c) pH at different experimental phases. Arrows indicate the sampling time for microbial analysis. [Adapted from **paper III**]

In summary, the three different studies performed in this chapter proved the possibility of acclimatizing normal AD microbiota into a specialised ammonia-tolerant methanogenic community. However, a certain amount of acclimatization time is needed. Aceticlastic methanogenic pathway was mainly responsible for the acetate utilization at high ammonia levels under both mesophilic and thermophilic conditions. Furthermore, it seems that the aceticlastic pathway was mainly mediated by *Methanosarcina* spp. Moreover, fed-batch acclimatization method was proved to be the most efficient method for ammonia-tolerant methanogenic consortia in terms of acclimatization time, TAN and FAN levels achieved and methanogenic activity. Finally, for the first time, acclimatization of methanogenic culture was proved to be possible in the CSTR reactors fed with natural protein-rich substrate. At the highest ammonia levels, *Methanosarcina* sp. was the most dominant methanogens

followed by *Methanoculleus* sp. The results extended the application of bio-augmentation technology in the future by developing the efficient method to acclimatize ammonia-tolerant consortia and elucidating the microbial characterization of these consortia.

3 Bioaugmentation as a method to improve methane production of ammonia inhibited reactors

Bioaugmentation has been widely and successfully used in many fields. In recent years, a few studies about using this technology to alleviate ammonia inhibition were also reported. For example, bioaugmentation with mixed culture of SAOB and hydrogenotrophic methanogens was performed on mesophilic CSTR reactors and up-flow anaerobic sludge blanket reactor (UASB) (Westerholm *et al.*, 2012, Fotidis *et al.*, 2013). However, both studies did not achieve the expected results, i.e. residual methane recovery and accumulated VFA consumption. The failure of these studies was mainly attributed to the slow growth of the ammonia-tolerant cultures and the restricted amount of bioaugmented microorganisms. Successful bioaugmentation to overcome ammonia inhibition was reported in another two studies, where mainly hydrogenotrophic methanogens were used (Fotidis *et al.*, 2014, Fotidis *et al.*, 2017). Both studies were performed in CSTR reactor fed with cattle manure under mesophilic conditions. On the one hand, bioaugmentation on reactors fed with natural protein-rich substrate, such as 3rd generation biomass microalgae, under extremely high ammonia levels, has never been reported before. On the other hand, successful bioaugmentation under thermophilic conditions was never studied. Moreover, the bioaugmentation working mechanism and the microbiological interaction between different microorganisms have never discussed.

Therefore, in this chapter, two bioaugmentation experiments were conducted to investigate the bioaugmentation effect on mesophilic and thermophilic CSTR reactors, respectively. Moreover, the mesophilic reactor was fed with 3rd generation biomass, i.e. protein-rich substrate microalgae. Additionally, microbial community changes before and after bioaugmentation were also followed in order to reveal the basic working mechanism.

3.1 Bioaugmentation with pure *Methanoculleus bourgensis* on a mesophilic CSTR reactor

In this study (paper IV), a CSTR reactor operated at extremely high ammonia levels (11 g NH_4^+-N L^{-1}) was used. The reactor was fed with mainly (80% in the feeding based on VS) 3rd generation biomass, microalgae *C. vulgaris*. An

inhibited steady state was observed at the first phase (P1). Then the bioaugmentation was performed with pure hydrogenotrophic *Methanoculleus bourgensis* during the second phase (P2) for four consecutive days. Microbial samples taken from before bioaugmentation (P1), immediately after bioaugmentation ($P3_a$) and three HRTs after bioaugmentation ($P3_b$, i.e. the end of the experiment), were used to elucidate the microbial dynamics during this process.

3.1.1 Improved methane yield and VFA reduction

The results clearly demonstrated a successful bioaugmentation effect. Specifically, the methane yield after bioaugmentation increased by around 30% compared to the inhibited steady state (P1). The methane improvement was in accordance with Fotidis *et al.* (2014), who reported a 31% methane increase after bioaugmentation of a reactor operated at TAN levels of 5 g NH_4^+-N L^{-1}. Moreover, the VFA levels decreased rapidly from more than 5 g L^{-1} to around 1 g L^{-1} immediately after bioaugmentation. A noteworthy observation was that propionate was the most dominant VFA during inhibited steady state, and it decreased from around 2.5 g L^{-1} to 0.1 g L^{-1} after bioaugmentation.

3.1.2 Establishment of a more efficient microbial community

The microbial analyses results showed that more than half of the interesting abundant OTUs changed significantly ($p<0.05$) after bioaugmentation (Fig. 5). Specifically, the bioaugmentation culture, *M. bourgensis*, increased its relative abundance from 3.6% to 6.7% after bioaugmentation. Considering the significantly improved reactor performance after bioaugmentation, it seems that even though *M. bourgensis* did not become the most dominant methanogen, its addition was sufficient to trigger the microbial community change towards to a more efficient community. This agreed with the reported "microbiological domino effect" (Fotidis *et al.*, 2014) that a bioaugmentation culture with low relative abundance still can cause the establishment of a new community. As a result, aceticlastic *M. soligelidi* increased its relative abundance from 36.3% to 66.1% after bioaugmentation. Meanwhile, the most dominant *Methanobrevibacter acididurans* before bioaugmentation decreased from 54.7% to 19.2% immediately after bioaugmentation and 4.9% at the end of the experiment.

The most abundant bacterial OTU before bioaugmentation was found to be SAOB *Syntrophaceticus schinkii* (Westerholm *et al.*, 2010). Moreover, another two OTUs were also assigned to SAOB: *Syntrophaceticus* sp. 33 and *Clostridium ultunense* 16 (Schnürer, 1996). In total, the SAOB changed in-

significantly between P1 and $P3_a$, but decreased significantly at $P3_b$. It indicated a stronger hydrogenotrophic activity during the period of before and immediately after bioaugmentation, compared to long-term period after bioaugmentation. This result was also coincided with the changes of aceticlastic and hydrogenotrophic methanogens during these periods.

Another remarkable OTU was *Bacteroidales* sp. 14, whose change after bioaugmentation had the most significant effect on the whole community changes. This OTU was similar to *Parafilimonas terrae* based on BLASTN results, which was reported to degrade propionate (Kim *et al.*, 2014). Moreover, *P. terrae* was phylogenetically close to *Filimonas*, who had a positive effect with propionate degradation (Albert *et al.*, 2016). Last but not the least, the uncultured SHA-98, which included several OTUs, increased its abundance after bioaugmentation from 20.1% to 26.4%. SHA-98 was reported to have the ability to tolerant high ammonia toxicity and degrade different complex organic matters (Mei *et al.*, 2016, Müller *et al.*, 2016). Thus its increase might result in a more efficient substrate utilization, which was in line with the improved methane production.

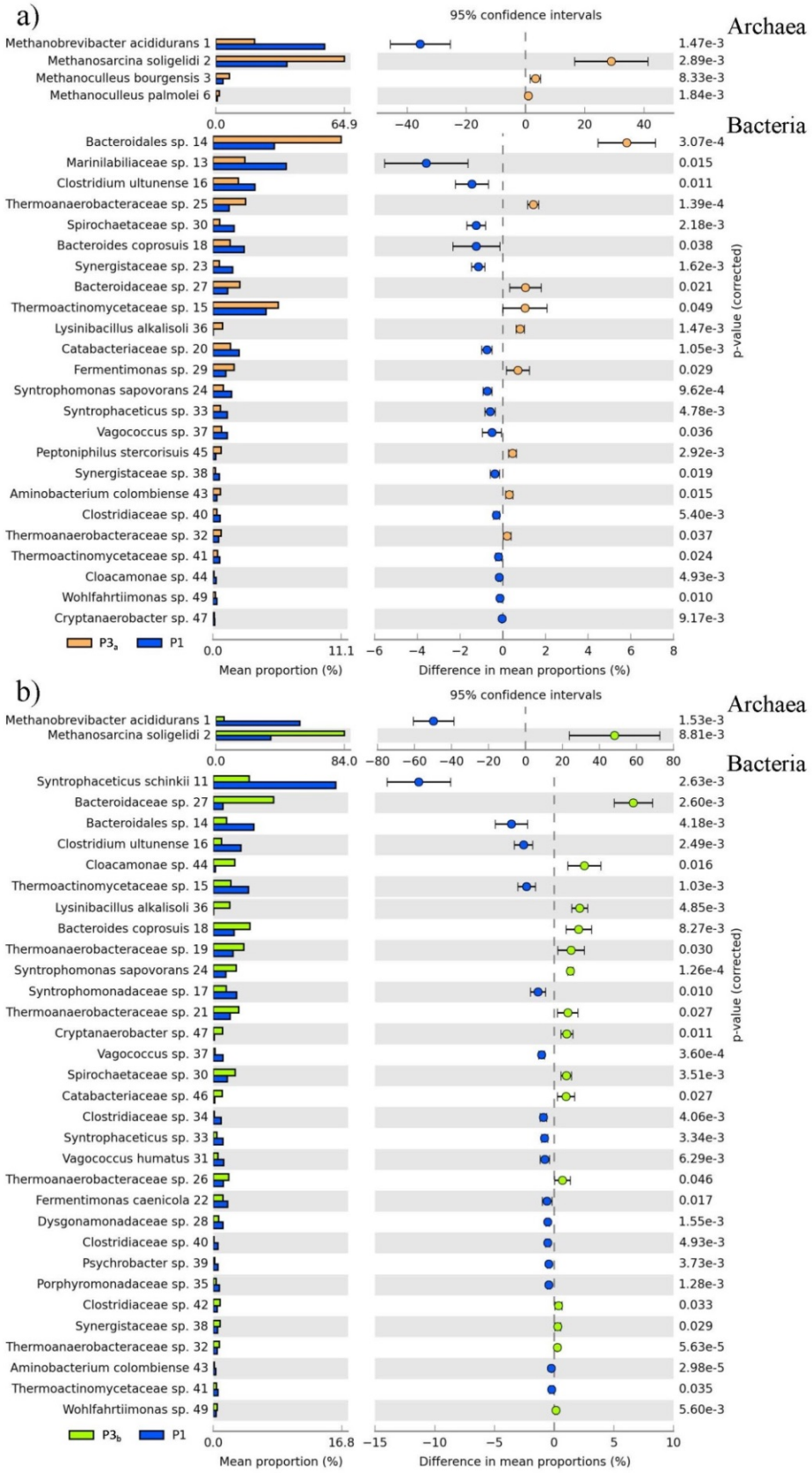

Figure 5. OTUs that changed significantly ($p<0.05$) among the most interesting OTUs a) P3a compared to P1, and b) P3b compared to P1. [Adapted from **paper IV**]

3.2 Different bioaugmentation strategies under thermophilic conditions

Thermophilic AD reactors have gained more and more attentions in these years due to some obvious advantages compared to mesophilic reactors. On the one hand, compared to mesophilic reactors, shorter HRTs and higher removal rate of the organic carbon were observed in thermophilic reactors (Weiland, 2010). On the other hand, a better destruction of the pathogenic bacteria was often achieved under thermophilic conditions (Kim *et al.*, 2002). However, FAN is the most toxic ammonia form and increases along with temperature. Thus thermophilic reactors usually encounter higher ammonia toxicity due to the higher temperature. Up to date, no successful bioaugmentation under thermophilic conditions has been reported. Therefore, this study (paper V) was aiming to achieve successful bioaugmentation on thermophilic reactors. Moreover, different bioaugmentation strategies with different bioaugmentation cultures were compared.

Table 5. Different bioaugmentation strategies for the CSTR reactors

	(Bio)Augmentation culture	(Bio)Augmentation dosage and frequency	(Bio)Augmented dry biomass
R_{ctrl}	BA medium and pure strain cultivation medium *	72mL each day three consecutive days	0
R_{enr}	Only enriched ammonia-tolerant methanogenic consortium	72mL each day three consecutive days	72 mg
R_{mix}	Mixed culture of pure hydrogenotrophic strain and the enriched consortium **	72mL each day three consecutive days	72 mg

* 50/50, based on volume; ** 50/50, based on volatile suspended solid

As shown in table 5, three different CSTR reactors were used in this study fed with cattle manure, and two bioaugmentation cultures were used: enriched ammonia-tolerant methanogenic consortium (T2 from paper I); mixed culture of pure hydrogenotrophic *Methanoculleus thermophilus* and the enriched consortium (50/50, based on volatile suspended solids). It must be mentioned that before mixing for bioaugmentation, the enriched consortium and the pure strain were cultivated separately at TAN levels of 5.0 g NH_4^+-N L^{-1} and pH of 8.0.

3.2.1 Recovered efficient reactor performance

During P1, the three reactors were operated under the same conditions with TAN levels below 2.1 g NH_4^+-N L^{-1}. The methane production of all the three reactors was similar ranging from 164-168 mL CH_4 g^{-1} VS (Fig. 6). However, after the TAN levels increased to 5.0 g NH_4^+-N L^{-1} at the beginning of P2, all

the reactors decreased their methane production immediately with 34-39% methane loss compared to P1.

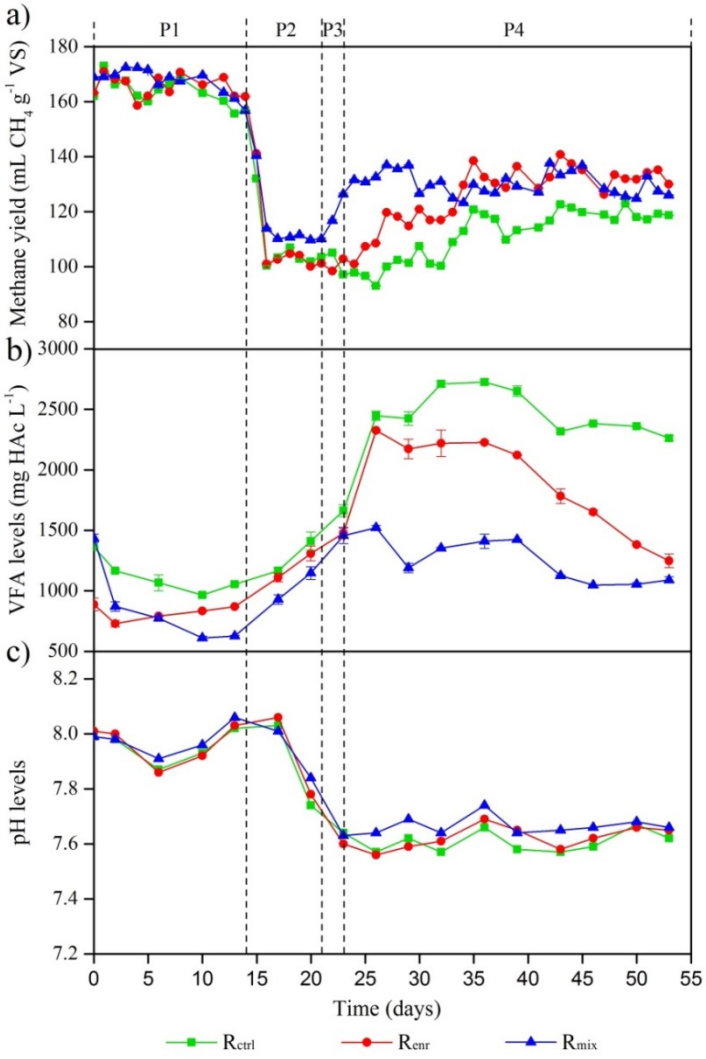

Figure 6. The performance of the three reactors at different experimental phases: a) methane yield, b) total VFA levels and c) pH. [Adapted from **paper V**]

Bioaugmentation were performed at P3. Immediately after bioaugmentation, the three reactors performed significantly differently. R_{mix} was the most efficient reactor since it responded to the bioaugmentation fastest and recovered the methane production to 78% compared to P1. R_{enr} also recovered the methane production similar to R_{mix}, but this happened ten days after bioaugmentation. In another word, R_{mix} produced 15% more methane than R_{enr} during the first ten days after bioaugmentation. Regarding R_{ctrl}, it also showed a gradually increasing trend of methane production, most probably due to microbial community adaptation (Calli *et al.*, 2005). However, the recovery efficiency of R_{ctrl} was significantly lower than the other two bioaugmented re-

actors. Overall, R_{mix} and R_{enr} produced 11-13% higher methane compared to R_{ctrl} after bioaugmentation, which for the first time demonstrated a successful bioaugmentation effect under thermophilic conditions.

The bioaugmentation effect was also supported by the difference of VFA levels in the reactors after bioaugmentation. After increasing the ammonia levels, the VFA levels increased up to 1500 mg L^{-1} at the end of P3 in all the reactors. However, the VFA levels in R_{mix} stopped increasing immediately after bioaugmentation, and fluctuated around 1000 mg L^{-1} in the rest of the experiment. The VFA levels in R_{enr} started decreased three days after bioaugmentation, and decreased down to around 1200 mg L^{-1} at the end of the experiment. On the contrary, the VFA levels in R_{ctrl} fluctuated between 2300 to 2700 mg L^{-1} after augmentation until the end of the experiment. Overall, the bioaugmentation effect resulted in 45-52% lower VFA levels in the bioaugmented reactors compared to the control.

The pH levels decreased by 0.3 units after bioaugmentation, and kept stable until the end of the experiment, which might be a balanced result between the accumulated VFA, ammonia levels and the buffer capacity of the manure. However, the pH was always within the suitable range.

3.2.2 Microbial community changes after bioaugmentation

Throughout the experimental period, *Methanothermobacter* sp. was the most dominant methanogen (Fig. 7), indicating a stronger hydrogenotrophic methanogenesis under thermophilic conditions. However, at the end of the experiment, the relative abundance of *Methanothermobacter* sp. decreased significantly in R_{mix}, while it kept stable in the other two reactors. The decreased *Methanothermobacter* sp. in R_{mix} was attributed to the bioaugmented *M. thermophilus*, evidenced by that the relative abundance of *M. thermophilus* increased more than two folds at the end. Considering the efficient reactor performance of R_{mix}, it was reasonable to emphasize the importance of *M. thermophilus* in the bioaugmentation mixed culture.

Another notable observation was that *M. thermophila* increased its relative abundance by more than two folds in R_{mix} and R_{enr} immediately after bioaugmentation, but not in R_{ctrl}. *M. thermophila* is the dominant methanogen in the enriched ammonia-tolerant methanogenic consortium. Thus the result indicated the successful delivery of targeted culture into the reactor through bioaugmentation. However, its high abundance in R_{mix} and R_{enr} did not keep until the end. Therefore, it seems that the bioaugmented *M. thermophila* only worked on the degradation of the accumulated acetate for a short period after

bioaugmentation (Hori *et al.*, 2006). However, it triggered the establishment of a new efficient microbial community supported by the good reactor performance after its disappearance at the end of the experiment.

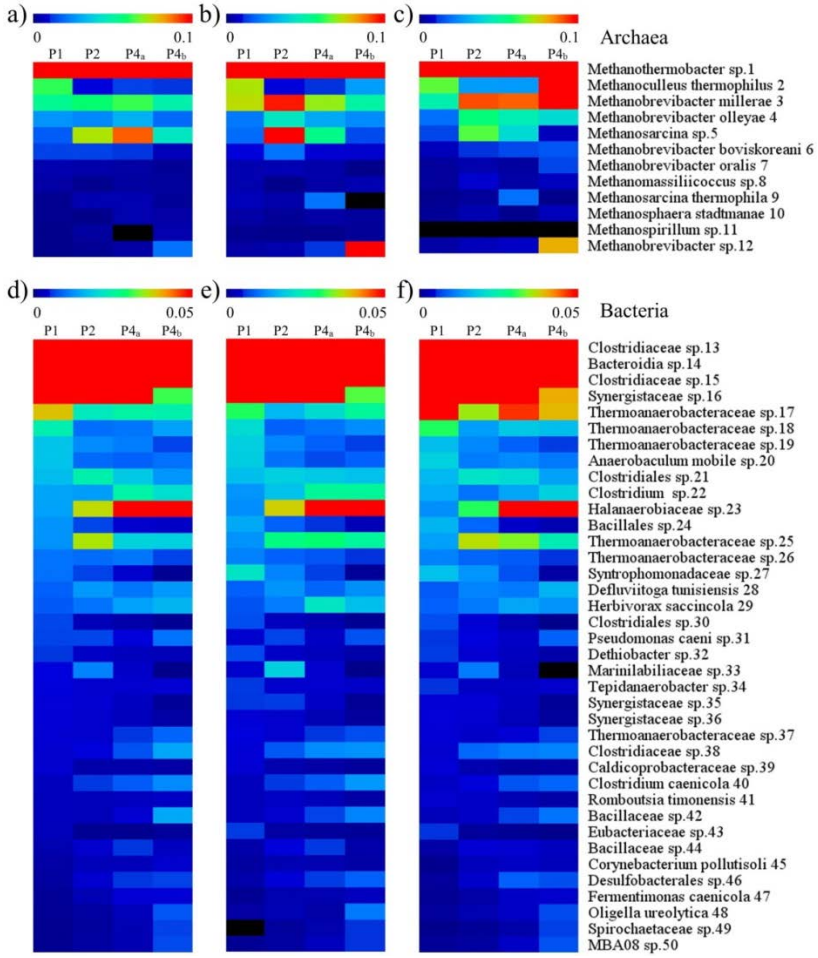

Figure 7. Relative abundance shown as heat map for the interesting archaea and bacteria at different phases in different reactors: a) archaea in R_{ctrl}, b) archaea in R_{enr}, c) archaea in R_{mix}, d) bacteria in R_{ctrl}, e) bacteria in R_{enr} and f) bacteria in R_{mix}. [Adapted from **paper V**]

The most interesting observation regarding the bacteria community was the changes of relative abundance of the well-known SAOB *T. phaeum* (Hattori *et al.*, 2000). To be specific, the relative abundance of *T. phaeum* decreased in all the reactors after increasing ammonia levels. However, immediately after bioaugmentation, *T. phaeum* increased significantly in R_{mix} resulting in a higher abundance compared to R_{ctrl} and R_{enr}. Therefore, it seems that the *M. thermophilus* in the mixed bioaugmentation culture enhanced the growth of SAOB, and consequently accelerated the SAO process and reduced the acetate levels.

Another noteworthy phenomenon was the change of relative abundance of the uncultured MBA08 after bioaugmentation. Among the interesting OTUs showing in Fig. 7, MBA08 consisted of four OTUs, including the most abundant OTU, i.e. *Clostridiaceae* sp.13, in all the microbial samples. The abundance of MBA08 increased significantly in R_{enr} (31% to 34%) and R_{mix} (28% to 35%) after bioaugmentation, but insignificantly in R_{ctrl} (30% to 31%). MBA08 was responsible for the degradation of cellulosic part in the manure (Sun *et al.*, 2015). Thus, it seems that the increased relative abundance of MBA08 positively correlated with the methane production of the reactors.

3.3 A proposed bioaugmentation mechanism

Overall, based on the two aforementioned bioaugmentation studies, bioaugmentation with either pure strain or enriched mixed culture can alleviate ammonia inhibition in AD process. It was proposed that the "microbiological domino effect" triggered by the bioaugmentation culture was the key mechanism that resulted in a successful bioaugmentation. Moreover, it seems that the instant reduction of hydrogen partial pressure by the bioaugmented hydrogenotrophic methanogens played an important role in overcoming ammonia inhibition.

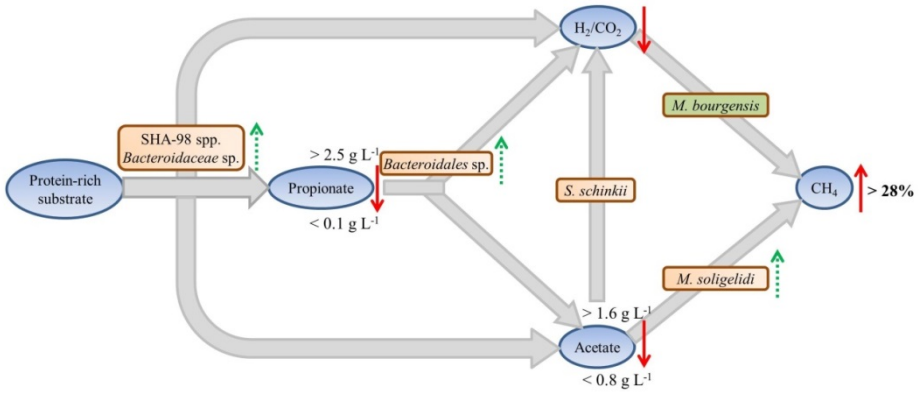

Figure 8. A schematic diagram depicting the proposed working mechanism of the bioaugmentation under mesophilic conditions. *M. bourgensis* was the bioaugmentation culture. Red solid and green dashed arrows present the change of the compound concentration and the OUT's relative abundance, respectively. [Adapted from **paper IV**]

Specifically, under mesophilic conditions, as shown in Fig. 8, the most possible explanation of the "microbiological domino effect" was attributed to the decreased hydrogen partial pressure by the bioaugmented *M. bourgensis*. The removal of hydrogen made the SAO process and other VFAs degradation thermodynamically favourable. Moreover, due to the domino effect, the corresponding microorganisms for these reactions also increased their relative

abundances, such as propionate degrader *Bacteroidales* sp. increased more than 100%. As a result, the overall VFA levels decreased rapidly, especially propionate levels. Additionally, the decreased VFA levels could also mitigate the reported synergistic inhibitory effect of ammonia and VFA (Lu *et al.*, 2013). Moreover, the bioaugmentation also triggered the increase of the microorganisms that are responsible for the upstream reactions, i.e. hydrolysis and acidogenesis, such as SHA-98 spp. The increased hydrolytic and acidogenic bacteria converted more original substrate into VFA, thus resulted in a higher methane production compared to the inhibited period. Therefore, an overall thermodynamically favourable condition formed after bioaugmentation and drove the whole microbial community towards to a more efficient AD process.

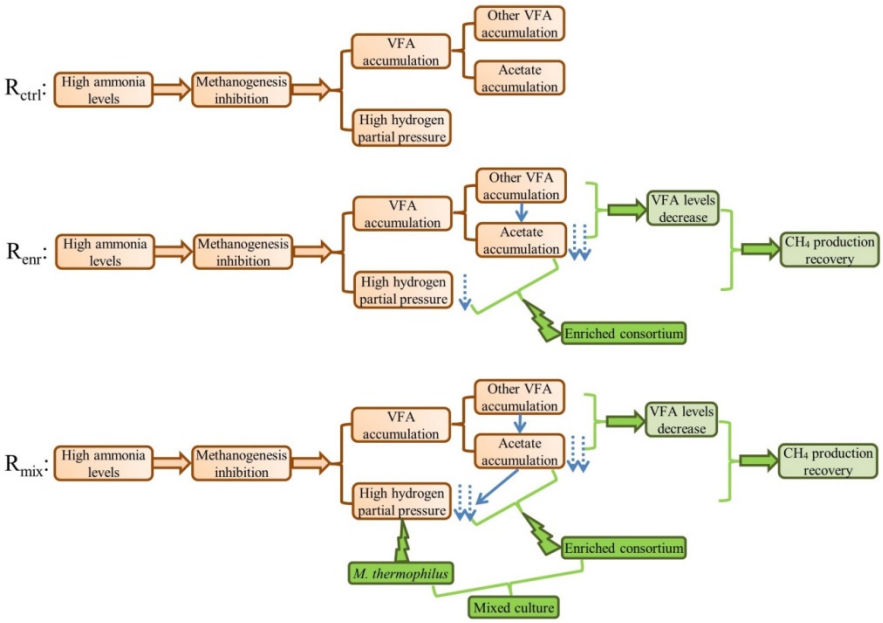

Figure 9. A proposed mechanism depicting the bioaugmentation effect under thermophilic conditions. The blue solid and dashed arrows present the conversion direction and decrease of the compounds' levels, respectively. The orange and green processes stand for the reactors' performance before and after bioaugmentation, respectively. [Adapted from **paper V**]

Under thermophilic conditions, both the two bioaugmentation cultures improved the reactor performance, and the mixed culture performed better than the enriched consortium. As shown in Fig. 9, a possible explanation for the better performance of the mixed culture was that *M. thermophilus* decreased the hydrogen partial pressure instantly. Similar to the mesophilic condition, the hydrogen removal triggered the increase of SAOB *T. phaeum*, and created favourable conditions for SAO process and acetogenesis of other VFA (e.g. propionate). Meanwhile, *M. thermophila* in the enriched consortium degraded

acetate directly. Thus, R_{mix} had the fastest methane recovery rate. However, when the reactor was bioaugmented with only enriched consortium (mainly aceticlastic *M. thermophila*), it seems that the consortium primarily decreased acetate levels. The enriched consortium didn't reduce hydrogen partial pressure as fast as the mixed culture. Thus, the enriched consortium recovered reactor performance eventually, but the recovery rate was relatively slower compared to the mixed culture.

In summary, the bioaugmentation experiments conducted in this chapter further confirmed the bioaugmentation effect on alleviating ammonia inhibition during AD process. For the first time, efficient AD process degrading protein-rich microalgae under extremely high ammonia levels was observed after bioaugmentation. Moreover, the first successful bioaugmentation under thermophilic conditions was also reported in this study with different bioaugmentation cultures. Overall, it was suggested that the instant hydrogen partial pressure reduction by efficient hydrogenotrophic methanogens (such as *M. bourgensis* and *M. thermophilus*) played an important role in overcoming the ammonia inhibition. The "microbiological domino effect" was proposed as the main working mechanism of bioaugmentation.

4 Ammonia inhibition influenced by other physicochemical factors

Ammonia is one of the most common inhibitors in AD process. However, there are other inhibitors, which might potentially combine with ammonia and result in a synergetic inhibitory effect. Moreover, except for other inhibitors, the ammonia derived from different ammonia sources might also result in different effects on the methanogens due to the different ways of introducing ammonia into the system. Therefore, in this chapter, two different experiments were performed to investigate the effect of another inhibitor (i.e. LCFA) and different ammonia sources (i.e. urea and ammonium chloride) on the ammonia inhibition of AD process.

4.1 Ammonia-LCFA synergetic co-inhibitory effect

In this study (paper VI), both batch and CSTR reactors were used to evaluate the potential ammonia and LCFA synergetic co-inhibitory effect under thermophilic conditions. Ammonium chloride was used as ammonia source, while sodium oleate was chosen to present LCFA.

Table 6. Experimental setup of different TAN and LCFA levels of the three batch reactor assays [Adapted from **paper VI**]

Assay I	Assay II	Assay III
TAN/LCFA (g NH_4^+-N L^{-1}/ g oleate L^{-1})		
2.60/0.00	2.60/0.00	4.00/0.05
4.00/0.00	2.60/0.05	4.00/0.20
5.00/0.00	2.60/0.20	4.00/0.50
7.00/0.00	2.60/0.50	4.00/1.00
	2.60/1.00	5.00/0.05
	2.60/2.50	5.00/0.20
	2.60/4.00	5.00/0.50
		5.00/1.00
		7.00/0.05
		7.00/0.20
		7.00/0.50
		7.00/1.00

In batch experiment, three different assays were set as shown in table 6: assay I was for individual ammonia test; assay II was for individual LCFA test; and assay III was for the combined co-inhibitory test. Likewise, in the continuous

conditions, also three reactors were used, which were initially fed with cattle manure (OLR=1.0 g VS L^{-1} d^{-1}). As shown in table 7, R_{TAN} was for individual ammonia test; R_{LCFA} was for individual LCFA; and R_{COM} was for the combined co-inhibitory test.

Table 7. Operational parameters in different experimental phases of the CSTR reactor experiment. [Adapted from **paper VI**]

Phases	Days	TAN/ LCFA (g NH_4^+-N L^{-1}/ g oleate L^{-1})		
		R_{TAN}	R_{LCFA}	R_{COM}
Phase 1 (P1)	0-12	2.60/0	2.60/0	2.60/0
Phase 2 (P2)	13-35	3.26/0	2.60/0.42	3.26/0.42
Phase 3 (P3)	36-56	3.82/0	2.60/0.77	3.82/0.77
Phase 4 (P4)	57-96	4.54/0	2.60/1.13	4.54/1.13
Phase 5 (P5)	97-143	5.58/0	2.60/2.18	5.58/2.18

The results from both batch and continuous reactors clearly demonstrated the existence of ammonia-LCFA synergetic co-inhibitory effect at specific LCFA and ammonia levels. Specifically, in batch reactors, the synergism was observed when the LCFA levels were equal or higher than 0.2 g oleate L^{-1} and the ammonia levels were in the range of 4.0 to 7.0 g NH_4^+-N L^{-1}. However, when the LCFA levels were too low (<0.2 g oleate L^{-1}) or ammonia levels were too high ($\geq$ 7.0 g NH_4^+-N L^{-1}), no synergism was observed. This might be explained by 1) no additional inhibition can be found if the toxicity was too low; and 2) all the AD steps, instead of only methanogenesis, were inhibited at extremely high ammonia levels ($\geq$ 7.0 g NH_4^+-N L^{-1}) (Lü *et al.*, 2008, Niu *et al.*, 2013), thus LCFA didn't intensify the overall inhibition.

In CSTR reactors, the synergetic effect was validated when LCFA and ammonia levels were higher than 1.1 g oleate L^{-1} and 4.5 g NH_4^+-N L^{-1}, respectively. Specifically, the methane inhibition in R_{COM} was higher than the sum of the inhibition in R_{TAN} and R_{LCFA} at the same ammonia and LCFA levels. Moreover, the VFA variations in the reactors further verified this synergism. For example, after increasing LCFA levels, a much faster VFA accumulation in R_{LCFA} was observed compared to R_{COM}, which indicated a slower LCFA degradation in R_{COM} due to the co-inhibitory effect. However, even with detected synergetic inhibition, adaptation to this synergism was also found, which was supported by the stable reactor performance with recovered methane production and accumulated VFA consumption after a long-term operation.

A possible mechanism for this synergism was that methanogenic inhibition mainly by high ammonia levels resulted in VFA and hydrogen accumulation, which slowed down the β-oxidation of LCFA by making β-oxidation process thermodynamically unfavourable (Lalman, 2000). This consequently brought about excess LCFA accumulation, which intensified the inhibition on the overall AD process.

4.2 Effect of different ammonia sources on pure methanogens

Many studies about ammonia inhibition in AD process were reported in the last three decades. However, the majority of these studies used ammonium chloride (NH_4Cl) as ammonia source to simulate the inhibition process. The advantage of using NH_4Cl is that it can release ammonium cations as soon as the addition into the solutions, thus create ammonia inhibition immediately. However, ammonia in a real anaerobic digester is usually from the degradation of protein, urea and nucleic acids. Moreover, urea is abundant in animal waste slurry and slaughterhouse wastewater (Møller *et al.*, 2004). Urea hydrolysis releases FAN directly, instead of ammonium cations as NH_4Cl. Therefore, it is interesting to investigate the potentially different effect from the different ammonia sources (i.e. NH_4Cl and urea) on the AD process, especially on the methanogenesis.

Therefore, in this study (paper VII), four different representative methanogens were used: mesophilic aceticlastic *Methanosarcina barkeri* MS DSM No. 800, thermophilic aceticlastic *Methanosarcina thermophila* TM-1 DSM No.1825, mesophilic hydrogenotrophic *Methanoculleus bourgensis* $MS2^T$ DSM No. 3045, and thermophilic hydrogenotrophic *Methanoculleus thermophilus* CR-1 DSM No. 2373. The ammonia inhibition test of each methanogen was performed with NH_4Cl and urea under different ammonia levels. The highest TAN levels were 10.0 and 5.0 g NH_4^+-N L^{-1} for mesophilic and thermophilic conditions, respectively.

The results showed that urea hydrolysis increased the pH of the medium to high levels (around 9.0), especially the medium for *M. barkeri*, *M. thermophila* and *M. bourgensis*. The high pH levels caused by urea hydrolysis were not suitable for methanogens to grow. On the contrary, NH_4Cl had a negligible effect on the pH. Additionally, the results also showed that a high buffer capacity of the medium, such as medium for *M. thermophilus*, could mitigate the pH increase to some extent.

In general, under the same ammonia levels, urea had a stronger inhibition on methanogens compared to NH_4Cl. As shown in table 8, except for the cases of *M. barkeri* and *M. thermophilus* at pH 7, urea showed higher inhibition up to 800% compared to NH_4Cl.

Moreover, heavier inhibition on aceticlastic methanogens compared to hydrogenotrophic methanogens was also observed at the same ammonia levels. For example, the highest inhibition was found less than 50% with both hydrogenotrophic methanogens, but almost 100% inhibition was found with the two aceticlastic methanogens, except for the case of *M. thermophila* with NH_4Cl.

Table 8. Overall comparison of highest methane production inhibition of all strains. [Adapted from **paper VII**]

Strains	pH	NH_4Cl	$CO(NH_2)_2$
***M. thermophila* ***	**7**	22.9 ± 0.9 %	91.0 ± 0.8 %
	8	57.9 ± 0.5%	98.5 ± 0.2 %
M. barkeri* *	**7**	99.4 ± 0 %	99.4 ± 0.1 %
	8	99.5 ± 0 %	99.6 ± 0.1 %
***M. thermophilus* ***	**7**	3.8 ± 2.7 %	0%
	8	28.7 ± 1.2 %	42.2 ± 6.6 %
***M. bourgensis* ***	**7**	3.1 ± 0.8 %	28.7 ± 1.2 %
	8	6.8 ± 0.7 %	15.2 ± 1.0 %

* For both pH levels, detected at 5 g NH_4^+-N L^{-1} for *M. thermophila* and *M. thermophilus*, and 10 g NH_4^+-N L^{-1} for *M. bourgensis*. ** For pH 7, detected at 7 and 5 g NH_4^+-N L^{-1} for NH_4Cl and urea, respectively; and for pH 8, both detected at 3 g NH_4^+-N L^{-1}.

The stronger inhibition of urea than NH_4Cl could be explained by their different way of introducing ammonia into the reactors. As discussed above, the direct hydrolytic product from urea is FAN, which is much more toxic than ammonium ions from NH_4Cl dissociation. Even though the ammonium ions and FAN will be eventually equilibrated driven by pH and temperature, urea still provides momentary high FAN levels. Additionally, without manual pH adjustment, urea hydrolysis increases pH to high levels. However, even with the pH adjustment in this study, completely avoiding the instant pH increase by urea hydrolysis was still not possible. Therefore, this momentary high FAN and pH levels during the urea hydrolysis, is proposed as the main reason for the higher inhibition from urea compared to NH_4Cl.

In summary, the two studies performed in this chapter demonstrated that other factors existing in the AD systems may influence the ammonia inhibition differently, such as other inhibitors and even different ammonia sources.

Specifically, an ammonia and LCFA synergetic co-inhibitor effect was identified in this chapter. The excess LCFA levels primarily due to high ammonia levels were proposed as a possible synergetic mechanism. Additionally, urea presented higher inhibition compared to NH_4Cl on pure methanogenic strains. However, further investigation using urea and NH_4Cl in a more complex AD process is still needed to have a more solid comparison.

5 Conclusions

This Ph.D. project focused on improving protein-rich substrate utilization by developing an innovative and reliable bioaugmentation technology to alleviate ammonia inhibition in anaerobic digestion (AD) process. Firstly, an extensive understanding about the ammonia-tolerant methanogenic consortia was achieved, including the growth rate, the microbial composition, the efficient acclimatization method, etc. Then successful bioaugmentation results were obtained under mesophilic conditions with microalgae as the main substrate and thermophilic conditions with manure as substrate. Additionally, the influence of long chain fatty acid (LCFA) and different ammonia sources on ammonia inhibition was assessed, respectively, which demonstrated the existence of ammonia-LCFA synergetic co-inhibitory effect and the different responses of pure methanogens to different ammonia sources. Specifically, the major conclusions from the Ph.D. project are summarized below:

- Acclimatization of methanogenic consortia to extreme high ammonia levels (up to 10.0 g NH_4^+-N L^{-1} and 1.4 g NH_3-N L^{-1}) was possible in batch reactors, together with an efficient μ_{max}.
- Aceticlastic pathway was the dominant acetate methanogenic pathway of the ammonia-tolerant consortia, which was mediated by *M. soligelidi* and *M. thermophila* at mesophilic and thermophilic conditions, respectively.
- Interestingly, methylotrophic *M. luminyensis* dominated in mesophilic ammonia-tolerant consortia. However, further investigation is needed to clarify the metabolic pathway of *M. luminyensis* found in this study.
- The fed-batch method was proved to be the most efficient method to acclimatise microbial consortia to high ammonia levels, compared to batch and continuous method.
- Successful methanogenic acclimatization up to 10.0 g NH_4^+-N L^{-1} was achieved in a continuously stirred tank reactor (CSTR) fed with mainly 3rd generation substrate microalgae, and *Methanosarcina* spp. was the most abundant methanogen.
- Syntrophic acetate oxidising bacteria *C. ultunense* and hydrogenotrophic *Methanoculleus* spp. increased their relative abundance during mesophilic CSTR acclimatization process, indicating an increased hydrogenotrophic activity at high ammonia levels.

- Bioaugmentation with hydrogenotrophic *M. bourgensis* improved 28% methane production of a mesophilic CSTR reactor fed with mainly natural protein-rich microalgae operated at total ammonia levels of 11 g NH_4^+-N L^{-1}.
- For the first time, successful thermophilic bioaugmentation was achieved, evidenced by 11-13% methane production improvement.
- Bioaugmentation with a mixed culture of pure hydrogenotrophic *M. thermophilus* and the enriched consortium had a faster methane recovery rate compared to only with the enriched ammonia-tolerant consortia.
- Instant hydrogen partial pressure reduction followed by a fast syntrophic acetate oxidation process and an accelerated acetogenesis process played an important role in alleviating ammonia inhibition.
- A "Microbiological domino effect" was proposed as the key mechanism of a successful bioaugmentation. The bioaugmentation culture is not necessary to be the most dominant species, but it can trigger the microbial community change towards to an efficient AD microbiota.
- Ammonia-LCFA synergetic co-inhibition effect was identified in both batch and continuous reactors. This synergism is proposed to be triggered by the excess LCFA levels due to β-oxidation inhibition by high ammonia levels.
- Urea showed much higher toxicity compared to NH_4Cl on pure methanogenic strains, mainly due to the higher momentary free ammonia levels and pH levels during urea hydrolysis.
- Hydrogenotrophic methanogens were more tolerant to ammonia toxicity compared to aceticlastic methanogens in pure strain cultivation condition.

6 Future perspectives

The present study developed an innovative bioaugmentation method to alleviate ammonia inhibition in AD process and improve the protein-rich substrate utilization efficiency. However, to further expand the fundamental knowledge about ammonia-tolerant consortia and apply bioaugmentation technology into full-scale AD process, several suggestions are given as follows:

- Isolation of *M. luminyensis* found in the mesophilic ammonia-tolerant methanogenic consortia should be done to further study its metabolic pathways at high ammonia levels, using more advanced microbiological analytical methods.
- A practical method to increase the biomass concentration of the bioaugmentation culture should be developed for the future commercial application, the same as the storage and transportation of the bioaugmentation culture.
- “Critical biomass”, which means the minimum amount of biomass for a successful bioaugmentation, needs to be investigated in order to reduce the overall cost of this technology.
- Bioaugmentation effect should be tested in different other scenarios to validate its effect, such as with high LCFA situation.
- Life cycle assessment and cost-benefit analysis should be performed in order to evaluate the environmental impacts and economic feasibility of this technology.

7 References

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8 Papers

I **Tian, H.,** Treu, L., Konstantopoulos, K., Angelidaki, I., Fotidis, I.A. 16s rRNA gene sequencing and radioisotopic analysis reveal the composition of ammonia acclimatized methanogenic consortia. *Submitted to Bioresource Technology*, 02 Aug 2018.

II **Tian, H.,** Fotidis, I.A., Mancini, E., Angelidaki, I., 2017. Different cultivation methods to acclimatise ammonia-tolerant methanogenic consortia. *Bioresource Technology* 232, 1-9.

III **Tian, H.,** Fotidis, I.A., Mancini, E., Treu, L., Mahdy, A., Ballesteros, M., González-Fernández, C., Angelidaki, I., 2018. Acclimation to extremely high ammonia levels in continuous biomethanation process and the associated microbial community dynamics. *Bioresource Technology* 247, 616-623.

IV **Tian, H.,** Mancini, E., Treu, L., Angelidaki, I., Fotidis, I.A. Bioaugmentation strategy for overcoming ammonia inhibition during biomethanation of microalgae. *Submitted to Renewable Energy*, 22 Aug 2018.

V **Tian, H.,** Yan, M., Treu, L., Fotidis, I.A., Angelidaki, I. Bioaugmentation as a trigger for the establishment of an efficient microbiota: focus on ammonia inhibition in thermophilic anaerobic digestion process. (*Manuscript under preparation for submission*)

VI **Tian, H.,** Karachalios, P., Angelidaki, I., Fotidis, I.A., 2018. A proposed mechanism for the ammonia-LCFA synergetic co-inhibition effect on anaerobic digestion process. *Chemical Engineering Journal* 349, 574-580.

VII **Tian, H.,** Fotidis, I.A., Kissas, K., Angelidaki, I., 2018. Effect of different ammonia sources on aceticlastic and hydrogenotrophic methanogens. *Bioresource Technology* 250, 390-397.

The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within five sections: Air, Land & Water Resources, Urban Water Systems, Water Technology, Residual Resource Engineering, Environmental Fate and Effect of Chemicals. The department dates back to 1865, when Ludvig August Colding gave the first lecture on sanitary engineering as response to the cholera epidemics in Copenhagen in the late 1800s.

Department of Environmental Engineering
Technical University of Denmark

DTU Environment
Bygningstorvet, Building 115
2800 Kgs. Lyngby
Tlf. +45 4525 1600
Fax +45 4593 2850

www.env.dtu.dk